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Cloning and characterisation of the chicken orthologue of dendritic cell-lysosomal associated membrane protein (DC-LAMP)

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ABSTRACT

A cDNA encoding the chicken orthologue of dendritic cell-lysosomal associated membrane protein (DC-LAMP)/CD208 was cloned by RT-PCR from RNA isolated from mature chicken bone marrow-derived dendritic cells (chBM-DCs). The cloned chicken DC-LAMP (chDC-LAMP) cDNA consists of 1281 nucleotides encoding an open reading frame of 426 amino acids (aa). Comparison of the deduced aa sequence of DC-LAMP with orthologous proteins from human and mouse revealed 27 and 24% identity, respectively. The predicted chDC-LAMP protein shares the characteristic features of LAMP family members. ChDC-LAMP mRNA, unlike its mammalian orthologues, was expressed in a wide range of tissues, at highest levels in the lung. Lymphoid tissues including thymus, spleen, bursa, ceacal tonsil and Meckel's diverticulum had high chDC-LAMP mRNA expression levels. ChDC-LAMP mRNA was expressed in all splenocyte subsets with the highest expression in Bu-1⁺ B cells and KUL01⁺ cells, which would include macrophages and DC. ChDC-LAMP mRNA was highly expressed in chBM-DC, whereas expression levels in chicken monocyte-derived macrophages (chMo-Mac) and the HD11 macrophage cell line were significantly lower. Following CD40L stimulation, chDC-LAMP mRNA expression levels were up-regulated in mature chBM-DC, chMo-Mac and HD11 cells whereas lipopolysaccharide (LPS) only up-regulated chDC-LAMP mRNA expression levels in chBM-DC. ChDC-LAMP is not solely expressed on chicken DC but can be used as a marker to differentiate between immature and mature DC.

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1. Introduction

Dendritic cells (DCs) are professional antigen-presenting cells with the unique function to activate primary immune responses [1,2]. Immature DCs, equipped with numerous receptors, are specialized in antigen capturing and processing. In mammals, upon activation by pathogen associated molecular patterns they undergo phenotypic changes and migrate into T-cell regions of secondary lymphoid tissues, where they complete their maturation and become specialized in presenting collected antigens to T cells to initiate adaptive immune responses. The phenotypes of DC in mammalian biomedical model species are well defined and some markers can be used to differentiate immature DC and

mature DC. Mature DC express up-regulated levels of major histocompatibility complex (MHC) class II and co-stimulatory molecules. The most specific markers for mature human DC are CD83 [3] and dendritic cell-lysosomal associated membrane protein (DC-LAMP) [4].

DC-LAMP/CD208 is a member of the LAMP family and is used as a specific marker of mature human, but not murine, DC [4,5]. In human DC, DC-LAMP is associated with the intracellular MHC II compartment and plays a role in the transfer of MHC class II molecules to the cell surface [4,6]. DC-LAMP is also used as a marker of mature DC in other mammals, including cattle [7] and pigs [8].

Chicken DC generated from bone marrow precursors has typical morphological, phenotypic and functional characteristics of DC [9]. Immature chicken bone marrow-derived DC (chBM-DC) showed high surface expression of MHC class II⁺ and putative CD11c, moderate or low levels of co-stimulatory molecules, and no surface expression of CD83 or DEC205. LPS- and CD40L-stimulated mature chBM-DC had elevated surface expression levels of co-stimulatory molecules (CD40 and CD86), CD83 and DEC205, compared to those seen on non-stimulated immature chBM-DC. In the hope of defining a novel marker for mature chicken DC, we have identified the chicken orthologue of DC-LAMP and investi-

Abbreviations: aa, amino acids; BM-DC, bone marrow-derived dendritic cells; ch, chicken; Chr., chromosome; CS, chicken serum; Ct, threshold cycle value; DCs, dendritic cells; DC-LAMP, dendritic cell-lysosomal associated membrane protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; hps, hours post-stimulation; hu, human; IL-4, interleukin-4; LPS, lipopolysaccharide; m, murine; MHC, major histocompatibility complex; Mo-Mac, monocyte-derived macrophages; PBS, phosphate-buffered saline.

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gated the expression patterns of chDC-LAMP in tissues, splenocyte subsets and immature and mature chBM-DC.

2. Materials and methods

2.1. Cloning of chicken DC-LAMP

The chDC-LAMP cDNA was predicted based on nucleotide sequence homology to human DC-LAMP sequences in Ensembl using BLAST (<http://www.ensembl.org/Multi/blastview>). Primers were designed according to the predicted sequence; forward primer (DC-LAMP F), 5'-ATGGGAAGGAGCAAATCACACT-3' and reverse primer (DC-LAMP R), 5'-TCAGATGTCCAAAAAGTCTT-3'. RNA from mature chBM-DC (see later) was used as RT-PCR template. First strand synthesis was for 50 min at 42 °C in a 20 µl volume containing 4 pmol of oligo dT, 200 U Superscript II (Invitrogen, Paisley, UK) and 500 ng chBM-DC RNA. After denaturation of the reverse transcriptase at 94 °C for 3 min, 2 µl of this reaction mix were added as template to a 20 µl PCR reaction, containing 20 pmol of each forward and reverse primer, 0.4 mM dNTPs, 1 µl Taq polymerase (Invitrogen). Cycling conditions were 30 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min.

2.2. Preparation of tissues and splenocyte subsets

Tissues and splenocyte subsets were obtained from three 6-week-old inbred line 7₂ chickens. The inbred line 7₂ originated from the Regional Poultry Research Laboratory (East Lansing, MI) and is a White Leghorn line maintained at the Institute for Animal Health (Compton, UK) [10,11]. Lymphoid tissues included thymus, spleen, bursa, Harderian gland, caecal tonsil, Meckel's diverticulum and bone marrow and non-lymphoid tissues included brain, muscle, heart, liver, kidney, lung and skin. Splenocyte subsets were isolated as described before [12,13]. Briefly, chicken spleens were digested with 556 µg/ml DNase I (Roche Diagnostics Ltd., Burgess Hill, West Sussex, UK) and 2.2 mg/ml collagenase D (Roche Diagnostics Ltd.) in Hank's Buffered Salt Solution for 1 h. Single cell suspensions were extracted using Histopaque 1077 (Sigma-Aldrich, Poole, UK) followed by three washes in PBS. 1 × 10⁸ cells were labelled with mouse anti-chicken CD4, CD8, Bu-1, TCR1, TCR2, TCR3, or KUL01 monoclonal antibodies (SouthernBiotech, Birmingham, Alabama, USA). Polyclonal goat anti-mouse IgG-coated microbeads (Miltenyi Biotec, Bisley, Surrey, UK) were used to isolate labelled cells by a standard positive selection (POSSEL) using an autoMACSpro separator (Miltenyi Biotec).

2.3. Culture of chicken bone marrow-derived dendritic cells, blood-derived monocyte-macrophages and the HD11 cell line

ChBM-DC was isolated as described previously [9]. Briefly, chicken bone marrow cells were cultured in the presence of recombinant chicken granulocyte-macrophage colony-stimulating factor (GM-CSF) and recombinant chicken interleukin-4 (IL-4). Recombinant chicken GM-CSF and IL-4 were produced from COS-7 cells transfected with pCIneo (Promega, Southampton, UK) expressing the relevant cytokine. There are no internationally agreed units of activity for avian cytokines. COS cell culture supernatants which contained chicken GM-CSF or IL-4 were both used at a dilution of 1:250 to maximise the number of cell aggregates. Immature chBM-DC aggregates started to grow from day 4. On day 6, LPS (*Escherichia coli* 055:B5, 200 ng/ml; Sigma-Aldrich) or CD40L (3 µg/ml) [14] was used to stimulate immature chBM-DC during a 48 h time-course. Non-stimulated or stimulated chBM-DC was harvested at different time-points for RNA preparation. Separate RNA preparations were isolated from chBM-DC from three different birds. RNA from chBM-

Table 1

Real-time quantitative RT-PCR probes and primers.

| RNA target | Probe/primer ^a sequence (5'-3') | Acc. No. |
|------------|--|----------|
| 28S | Probe (FAM)-AGGACCGCTACGGACCTCCACCA -(TAMRA) F GGCGAAGCCAGAGGAAACT R GACGACCGATTGACACGTC | X59733 |
| DC-LAMP | Probe (FAM)-TCTGCGCATTTCTTCTGCTGTGC -(TAMRA) F CACACTTGGTCTTGCTAGCCTTT R TGACACCCAGGGCCACTT | AM933592 |

^a F = forward primer; R = reverse primer.

DC stimulated with LPS for 24 h was used as template to clone chDC-LAMP cDNA.

Chicken monocyte-derived macrophages (chMo-Mac) were isolated from peripheral blood of three 6-week-old inbred line 7₂ chickens. Ten millilitres of blood (with K₃EDTA as anti-coagulant) were diluted with PBS and centrifuged over Histopaque 1083 (Sigma-Aldrich) at 1200 × g for 40 min. The interface was collected, washed and adjusted to 5 × 10⁶ cells/ml in RPMI 1640 (Invitrogen) with 5% chicken serum (CS) (Invitrogen). Cells were cultured in 6-well-plates at 41 °C, 5% CO₂ overnight and non-adherent cells were gently removed. Adherent cells were incubated for a further 24 h and then stimulated with LPS or CD40L for another 24 h.

The HD11 avian macrophage-like cell line [15] was cultured in RPMI 1640 (Invitrogen) containing 2.5% foetal calf serum (Invitrogen), 2.5% CS (Invitrogen), 10% tryptose phosphate broth, 20 mM L-glutamine, 1 U/ml penicillin and 1 mg/ml streptomycin. HD11 cells were trypsinised, seeded to a 6-well-plate and then stimulated with LPS or CD40L for 24 h. Unstimulated or stimulated chMo-Mac and HD11 cells were harvested for RNA preparation.

2.4. Total RNA isolation

RNA from the tissues and cells isolated and cultured as described above was extracted using an RNeasy Mini kit (QIAGEN, Crawley, UK) following the manufacturer's instructions.

2.5. Real-time quantitative RT-PCR analysis of chDC-LAMP expression

ChDC-LAMP mRNA levels in different tissues and cells were quantified using TaqMan real-time quantitative RT-PCR (qRT-PCR), a well-described method [13,16–18]. ChDC-LAMP-specific primers and probe (Table 1) were designed using the Primer Express software program (Applied Biosystems, Warrington, UK). The probe lies across the boundary between exons 1 and 2. Primers and probe for 28S RNA have been described previously but for clarity are also given in Table 1. The assays were performed using the TaqMan Fast Universal PCR master mix and one-step RT-PCR master mix reagents (Applied Biosystems). Data are expressed in terms of the threshold cycle value (Ct). The chDC-LAMP-specific product for each sample was normalised using the Ct value of 28S rRNA product for the same sample. Final results are shown either as 40-Ct, using the normalised value, or as fold difference from levels in control chBM-DC.

3. Results and discussion

3.1. Cloning and sequence analysis of chDC-LAMP

In human, the DC-LAMP gene is encoded on chromosome 3 (Chr. 3). We used the huDC-LAMP sequence to search the chicken

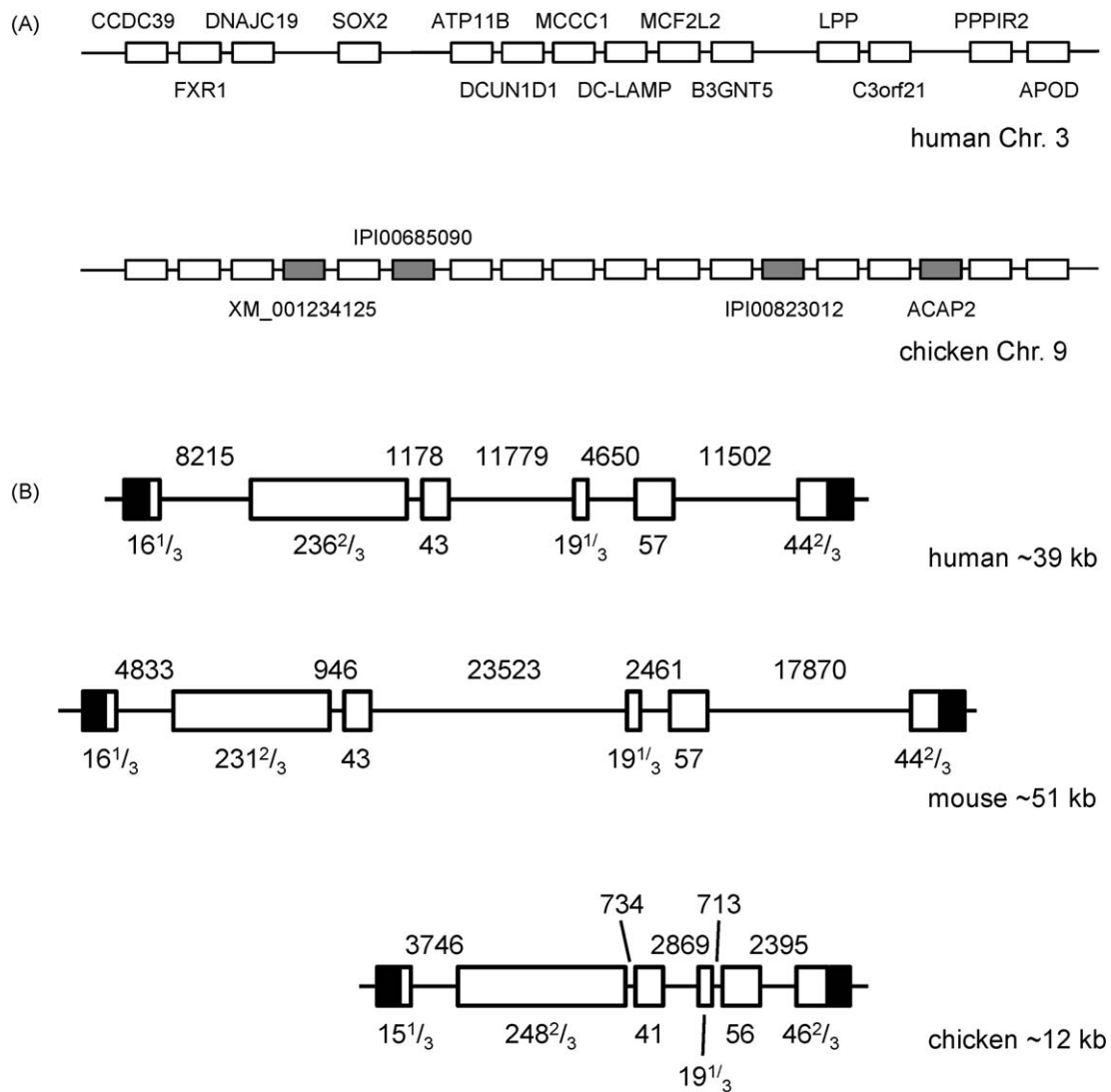


Fig. 1. (A) Conserved synteny between human Chr. 3 and chicken Chr. 9, around DC-LAMP. Genes are shown as boxes, intergenic regions as the lines joining the boxes, neither being to scale. The human genes are labelled centred above or below the boxes. The same genes are present in the same order in the chicken as in human, with an additional four chicken genes (labelled and shaded) for which there are no obvious human orthologues. (B) Comparison of the gene structures of human (Acc. No. AJ005766), mouse (Acc. No. NM_177356) and chicken (Acc. No. AM933592) DC-LAMP. Exons are shown as boxes; black boxes represent the UTRs (length unknown in the chicken), open boxes coding sequence; introns are shown as lines joining the boxes. Numbers represent exon (below) and intron (above) lengths in aa and base-pairs, respectively.

genome in Ensembl (by BLAST) and predicted the cDNA sequence of the chicken homologue of huDC-LAMP. The gene encoding chDC-LAMP lies on Chr. 9, in a group of genes with conserved synteny with human Chr. 3 (Fig. 1A). The full length cDNA of chicken DC-LAMP consists of 1281 nucleotides encoding an open reading frame of 426 amino acids (aa) (Acc. No. AM933592). The gene structure of chDC-LAMP is similar to those of human and mouse DC-LAMP, comprising six exons and five introns, with similar numbers of aa encoded by the respective exons across species (Fig. 1B). Overall, the gene is less than one-third the length of huDC-LAMP and one-fourth that of mDC-LAMP.

The predicted chDC-LAMP polypeptide has the typical features of a type I transmembrane protein. It has a presumptive signal peptide of 22 aa, as predicted by the SignalP program (<http://www.cbs.dtu.dk/services/SignalP>), followed by a long extra-cellular domain (369 aa), a hydrophobic transmembrane domain (23 aa), and a short cytoplasmic domain (12 aa), as predicted by the TMHMM2 program (<http://www.cbs.dtu.dk/services/TMHMM-2.0>). ChDC-LAMP also shares characteristic features of LAMP family members [19,20] (Fig. 2A). Like human

and mouse DC-LAMP, the luminal domain of chDC-LAMP is divided by a serine/proline-rich region. The membrane-proximal domain contains four conserved cysteines which have the potential to form two disulphide bridges. The short C-terminal cytoplasmic tail contains a conserved tyrosine-based motif (Tyr-X-X-hydrophobic residue) for lysosomal targeting [21–23]. ChDC-LAMP is also rich in N-glycosylation sites (11 in total). A phylogenetic tree (Fig. 2B) illustrates the conservation of aa sequences of LAMP proteins between different species. The tree indicates that chDC-LAMP is a new member of the chicken LAMP family and is closer to mammalian DC-LAMP than chLAMP1 or chLAMP2. Comparison of the deduced aa sequence of chDC-LAMP with its human and mouse orthologues revealed 27 and 24% identity, respectively.

3.2. Expression of chDC-LAMP in different tissues and splenocyte subsets

Expression of chDC-LAMP mRNA in lymphoid and non-lymphoid tissues and splenocyte cell subsets was assessed by

(A) human : -MPROL S A A A A L F A S L A V I L H D G S O M R A K A F P E T R D Y S Q P T A A A T V Q D I K K P V Q Q P A K Q A
mouse : -M P G Q I S A V A V L F L S L T V I L H - G Y Q I R E K E F P K A R G Y L Q Y T A T S A E Q I T T K P L L Q L I N Q R
chicken : M G R S K S H L V L L A F V C A F S S C C A E V A L G V R L S P Q T T S F H H T I T S A L P L S V Y H S P P H L S T T V

human : P H Q T L A A R F M D G H I T ----- F Q T A A T V K I P T T P A T T K N T A T T S P I T Y T L V T T Q A T P N
mouse : S H I T L A S R F K D D Y I Q M A A E T S A I E N T A H I T M K T V T P V T T K S L P P I S S A S Y T F V R S ---- N
chicken : Q P N T T G S I S H T T T L Q T T D Q H W V T T A P A S H M T T Q A G A N T S K A H G Q T S S T A V T T T A A D T A A A

human : N S H T A P P V T E V T V G P - S L A P Y S L P P T I T P P A H T T G T S S T V S H T T G N T T Q P S N Q T T L P A T
mouse : N A H M T A S S T D D T I G S G S I A H L P V P ----- T T R A S L A I V N Y I T G R A T Q L G G Q T T L P K T
chicken : G Q A T T Q A M E T V T Q A V K N V T V P P Y N Q I T T H V D T V T N T T I E N T T S K T Q T T T A T N T T A T T S S

human : L S I A L H K S T T G Q K P -- V Q P T H A P G T T A A A H N T T R T A A P A S T V P G P T L A P Q P S S V K T G I Y Q
mouse : F F T A S H K S T T N Q R P -- T L S T N V L G T S T P T H K D R S T T S P V P L V P R P T L V T W S S P A K I G T Y E
chicken : T V K P T T S S T N H T T S G T S A T T M T N A T T T H Q G T H T T I P S T T M M V R P T L A P Q P S P I P T G T Y I

human : V L N G S R L C I K A E M G I Q L I V Q D K E S V F S P R R Y F N I D P N A T Q A S G N C G T R K S N L L L N F Q G G F
mouse : V L N G S R L C I K A E M G L A L I V Q E K D L D S A T Q R Y F N I D P S L T H A S G K C D S Q K S N L F L N F Q G G S
chicken : I S S S N K T C I K A V M G L Q L M A L S T Q -- K Q M K Y L T V S P N A T Q I S G S C G M V Q S V L N I T F P G G F

human : V N L T F T K D E E S Y Y I S E V G A Y L T V S D P E T I Y Q G I K H A V V M F Q T A V G H S F K C V S E Q S L Q L S A
mouse : V N I T F T K E N L Y Y I S E V G A Y L T I S N T E K T Y Q G K K N T L M M F E T V V G H S F K C V S E Q S I Q L S A
chicken : I S F V F V K D P T Y Y V S T I E A E L Q L P S E G I L Y Y - V A I R Q Q H F T A K L G N S Y K C A S K Q T F G L E R

human : H L Q V K T T D V Q L Q A F D F E D D H F G N V D E C S S D Y T -- I V L P V I G A I V V G L C L M G M G V Y K I R L R
mouse : Q L Q M K T M N I H L Q A F D F E G D S F G N V N E C L S D Y T -- V V L P M V A I I V V V I C V V G L S V Y K I R Q R
chicken : T Y Q L V I V N M Q L Q A F D I V D N Q F G R E E C F L D K S T K A V P V A V G L S I L G L L V I V F V T F E L I S R R

human : C Q S S G Y Q R I
mouse : H Q S S A Y Q R I
chicken : K P Y R G Y E R I

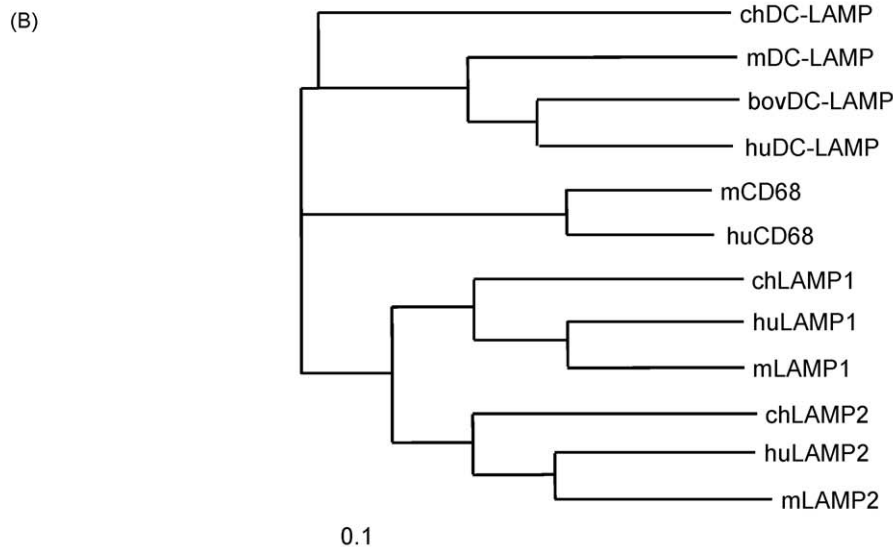


Fig. 2. (A) Alignment of the predicted aa sequence of chicken DC-LAMP (Acc. No. AM933592) with its human (Acc. No. AJ005766) and mouse (Acc. No. NM_177356) orthologues. Shaded areas represent conservation of aa similarity. Gaps introduced for optimal alignment are indicated by dashes. The potential N-glycosylation sites in chDC-LAMP are underlined. The conserved cysteines are indicated with asterisks. The conserved serine/proline-rich region is underlined with a dotted line. The conserved

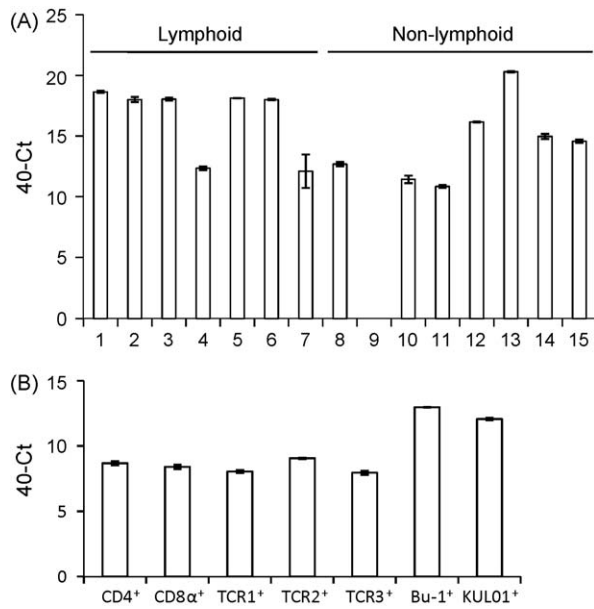


Fig. 3. Expression patterns of chDC-LAMP mRNA, as measured by real-time qRT-PCR, with results expressed as 40-Ct values ± SE. A. Expression in chicken lymphoid (1–) and non-lymphoid (8–15) tissues; 1, thymus; 2, spleen; 3, bursa of Fabricius; 4, Harderian gland; 5, caecal tonsil; 6, Meckel's diverticulum; 7, bone marrow; 8, brain; 9, muscle; 10, heart; 11, liver; 12, kidney; 13, lung; 14, skin; 15, small intestine. (B) Expression in chicken splenocyte subsets.

real-time qRT-PCR (Fig. 3A). ChDC-LAMP mRNA was constitutively expressed in all the lymphoid and non-lymphoid tissues tested except for muscle. Amongst lymphoid tissues, chDC-LAMP mRNA was most highly expressed in the thymus, spleen, bursa, caecal tonsil and Meckel's diverticulum. In non-lymphoid tissues, it was most highly expressed in the lung. In human, mouse and sheep, DC-LAMP mRNA is highly expressed in the lung and is described as a marker of adult type II pneumocytes in those species [4,5,24,25]. In human, lymphoid organs are major sources of DC-LAMP mRNA [4]. However, in the mouse, only lymph nodes and the spleen, other than the lung, express DC-LAMP mRNA, at very low levels, contrasting with the expression of DC-LAMP mRNA in human [5] and the chicken.

In human, DC-LAMP mRNA is specifically expressed in DC and is up-regulated after DC maturation and activation. Among freshly isolated cells, DC-LAMP is only present in resting or phorbol myristate acetate-ionomycin-activated DC, but not in activated monocytes, T cells, granulocytes, peripheral blood lymphocytes and B cells [4]. However, chDC-LAMP mRNA was detected in all splenocyte subsets (Fig. 3B), although for CD4⁺, CD8⁺, TCR1⁺, TCR2⁺ and TCR3⁺ subsets, the expression levels were comparatively and significantly lower than in Bu-1⁺ and KUL01⁺ splenocytes. Chicken Bu-1 is a marker for chicken B cells and is also expressed on subsets of macrophages and monocytes [26,27]. The monoclonal antibody KUL01 identifies chicken monocytes and macrophages as well as interdigitating cells and activated microglia cells and is considered to be a marker for chicken monocytes and macrophages [28,29]. Our unpublished data also suggest that it recognises chBM-DC. Our results suggest that unlike mammalian DC-LAMP, chDC-LAMP mRNA may also be expressed in chicken B cells and macrophages.

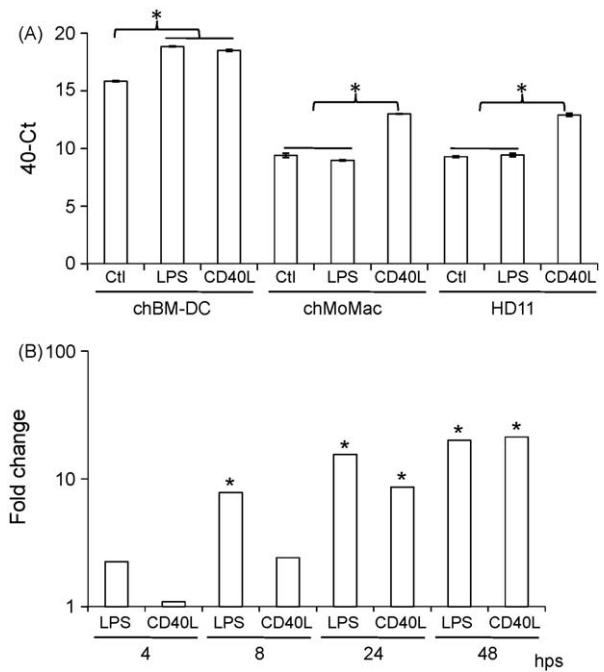


Fig. 4. (A) Expression patterns of chDC-LAMP mRNA, as measured by real-time qRT-PCR, with results expressed as 40-Ct values ± SE, in chBM-DC, chMo-Mac and HD11 cells which were unstimulated (Ctl) or stimulated with LPS or CD40L for 24 h. (B) Expression patterns of chDC-LAMP mRNA, as fold-change from levels in time-matched unstimulated (Ctl) chBM-DC, in chBM-DC stimulated with LPS or CD40L during a time-course. hps = hours post-stimulation. (*) Levels are statistically significantly different at $p < 0.05$ when compared with levels in unstimulated (Ctl) chBM-DC.

3.3. Expression of chDC-LAMP in chicken DC and macrophages

ChDC-LAMP mRNA expression levels were measured in *in vitro* cultured chBM-DC, *ex vivo* chMo-Mac and HD11 cells (Fig. 4A). ChDC-LAMP mRNA was highly expressed in unstimulated chBM-DC but also significantly up-regulated after stimulation with LPS or CD40L for 24 h. As characterised recently [9], unstimulated chBM-DC are primarily immature DC, and LPS and CD40L drive chBM-DC maturation. Similarly, human DC-LAMP mRNA is up-regulated after DC maturation and activation [4,30]. When compared with chBM-DC, DC-LAMP mRNA expression levels in chMo-Mac and HD11 cells were significantly lower. CD40L, but not LPS, stimulation up-regulated the expression of chDC-LAMP mRNA in chMo-Mac and HD11 cells.

Fig. 4B shows the mRNA expression pattern of chDC-LAMP in chBM-DC during a time-course after stimulation with LPS or CD40L. ChDC-LAMP mRNA was significantly up-regulated after stimulation with LPS after 4 h and with CD40L after 12 h. The up-regulation continued until 48 h post-stimulation, by which time-point most chBM-DC in similar stimulation assays were mature [9]. The kinetics of up-regulation of chDC-LAMP mRNA after maturation of chBM-DC were similar to those for DC-LAMP mRNA in human CD34⁺ cell-derived DC and monocyte-derived DC [4,30]. However, our results were in contrast to the expression pattern of DC-LAMP in mouse DC, in which DC-LAMP was never detected, no matter the source of cells (*in vitro*-derived, *ex vivo*-purified or *in vivo*) or the type of activation [5]. Our results on cell and tissue expression suggest that chDC-LAMP may not be a specific marker

for chicken DC, although this needs more thorough investigation. However, its expression is up-regulated on mature vs. immature DC, suggesting that, as in human, it can be used as a marker to differentiate between antigen-sampling and antigen-presenting DC.

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